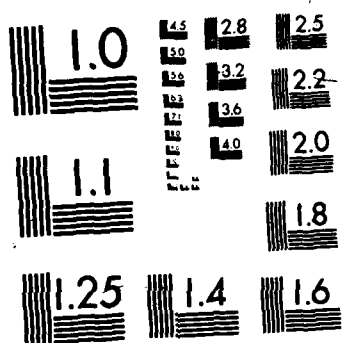


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ANNUAL PROGRESS REPORT

CHARACTERIZATION OF THE VARIABLE ANTIGEN GENES  
EXPRESSED BY TRYPANOSOMA BRUCEI RHODESIENSE  
DURING METACYCLIC STAGE AND IN THE BLOODSTREAM

JOHN E. DONELSON

September 1, 1983

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND

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University of Iowa  
Iowa City, Iowa 52242

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)  <b>Two different cDNA libraries have been constructed using mRNAs extracted from "day 5" trypanosomes which have been shown to be expressing predominately metacyclic variable surface glycoproteins (metacyclic VSGs). These cDNA libraries have been screened via the +/- hybridization procedure for those clones which possess metacyclic VSG coding sequences. Five different VSG cDNAs have been identified to date and the complete, or nearly complete,</b>		

## 20. Abstract - continued

sequence of each has been determined. Two of the five cDNAs are non-overlapping and appear to code for a specific metacyclic VSG called MVAT 7 which is defined by monoclonal antibodies. (Klaus Esser of Walter Reed Army Institute of Research, personal communication). We are in the process of performing genomic DNA Southern blots to see if the MVAT 7 VSG gene undergoes rearrangements when it is expressed in trypanosomes as do bloodstream VSG genes. In addition we are constructing vectors suitable for expression of this VSG coding sequence in bacteria so that a sufficient quantity can be obtained for vaccination studies. As a corollary to the metacyclic VSG gene project, we are also characterizing VSG genes expressed during the trypanosome bloodstream stages. We have determined the boundary sequences of an expression-linked extra copy VSG gene and demonstrated that this gene remains in the genome when the trypanosome clone is placed in procyclic culture conditions where the VSG is no longer made. These projects will be continued and expanded in the coming year if the contract is renewed.



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## ANNUAL PROGRESS REPORT

CONTRACT NO. C-2228

"Characterization of the Variable Antigen Genes  
Expressed by Trypanosoma brucei rhodesiense  
During Metacyclic Stage and in the Bloodstream"

September 15, 1982 - September 1, 1983

John E. Donelson, Ph.D., Principal Investigator  
Dept. of Biochemistry  
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Iowa City, Iowa 52242

Two general research projects were initiated during this first year of Contract No. C-2228. They are described here under the headings of (A) The Metacyclic VSG Gene Project and (B) The Bloodstream VSG Gene Project. Good progress has been made on both projects. No publications have appeared yet but as summarized below, two major manuscripts have been submitted, but not yet accepted, on the bloodstream VSG project and we will soon be ready to prepare our first manuscript on the metacyclic VSG project. In reality both projects are just beginning and we feel we are now positioned to make some very interesting contributions to the area of the trypanosome antigenic variation during the coming grant period.

### A. The Metacyclic VSG Gene Project.

Much of the effort during this first year of the research grant was devoted to preparing the metacyclic trypanosome cDNA libraries and screening them for bacterial clones that contain cDNAs for the metacyclic VSGs (M-VSGs). The starting material for this work has been "day 5 metacyclic trypanosomes" which are supplied to us by Klaus Esser and his colleagues at Walter Reed Army Institute of Research. These trypanosomes are isolated from mice 5 days after infection with metacyclic trypanosomes that have been prepared from tsetse fly salivary glands. Klaus Esser and his colleagues have shown that the majority of these "day 5 trypanosomes" are still expressing the VSGs present on the true metacyclic trypanosomes. Then at day 5, or shortly thereafter, there is a dramatic switch to the expression of bloodstream VSGs which are antigenically distinct from the M-VSGs. The use of the "day 5 trypanosomes" represents a compromise in the choice of starting material. It would, of course, be better to start with actual metacyclic trypanosomes from the tsetse salivary gland. However, each fly yields only  $10^4$ - $10^5$  trypanosomes (Klaus Esser, personal communication) and  $10^9$  organisms are required to obtain sufficient amounts of RNA and DNA for the molecular biology techniques. Since it is not practical to dissect out the salivary glands of 10,000 flies to obtain the  $10^9$  trypanosomes, we must use experimental animals as the reservoir in which to amplify the number of organisms up to the necessary quantity. We recognize that this might eventually introduce some difficulties in interpreting genomic Southern data (for example, perhaps the M-VSGs are expressed via ELC genes that are deleted from the genome before day 5) but that is the constraint we must contend with in this system. Nevertheless, since the monoclonal antibodies (see below) are clearly directed against M-VSGs, there should be no doubt about the experiments which utilize these monoclonal antibodies. And in fact the vast majority of the planned experiments are not affected by the fact that the trypanosomes are from "day 5" rather than from the tsetse fly.

Table 1 below summarizes the distribution of M-VSGs (MVAT antigens) in four of the day 5 trypanosome populations that we have used to isolate mRNA for constructing and screening the cDNA libraries.

MVAT antigen	PREPARATIONS OF METACYCLIC ORGANISMS			
	MVAT Mix	87% MVAT 7	MVAT 6 <sup>-</sup>	MVAT 7 <sup>-</sup>
1	5.3%	-	-	-
2	11.8	-	-	-
3	5.4	-	0.5%	1.0%
4	7.0	-	1.0	1.0
5	1.3	-	0.5	0.5
6	48.0	1.0%	-	69.0
7	15.0	87.0	47.0	-
8	12.0	-	-	-
9	1.1	-	2.0	1.0
10	0.4	-	3.0	7.0
11	2.4	-	-	-
12	3.3	-	-	-
13	-	2.0	2.0	3.0
14	-	-	10.0	7.0
TOTAL	113%	90%	66%	89.5%

Table 1. Distribution of M-VSGs (MVAT antigens) in four different preparations of day 5 trypanosomes.

A total of 14 different M-VSGs have been identified by monoclonal antibody screening (Klaus Esser, personal communication). The MVAT MIX population shown in Table 1 can be thought of as a "wild type" representation of M-VSGs in the LVH 18 metacyclic lineage. This is a typical distribution of M-VSGs in wild type metacyclic populations. The percentage of a given M-VSG in the population is detected by fluorescent monoclonal antibody binding to the trypanosomes. For example, 5.3% of the MVAT MIX population react with monoclonal antibody against M-VSG 1 and 15% react with antibody against M-VSG 7 (Table 1). The population called 87% MVAT 7 is one in which an actual metacyclic population was neutralized with antibodies to all of the M-VSGs except M-VSG 7 before injection into experimental animals. Therefore the day 5 population is greatly enriched for M-VSG 7. In this case 87% of the population reacted with M-VSG 7 antibody while 1% and 2% reacted with M-VSG 6 and M-VSG 13 antibody respectively. Only 90% of the population reacted with the total battery of metacyclic monoclonals which probably means that 10% of the day 5 popu-

lation had switched to bloodstream VSGs. The MVAT 6<sup>-</sup> and MVAT 7<sup>-</sup> day 5 populations have been specifically depleted of M-VSG 6 and 7 respectively by neutralizing the metacyclic trypanosomes with antibodies against these M-VSGs before injection into experimental animals.

We have used the procedure of Land et al (1) to prepare two metacyclic cDNA libraries. One is from the mRNA of the MVAT MIX population. The other is from mRNA of another day 5 population that is enriched in M-VSG 7 similar to 87% MVAT 7 (and called 70% MVAT 7). About 1000 clones are in the MVAT MIX library and 700 are in the M-VSG 7 enriched library. These 1700 clones are stored individually in microtitre dishes. The procedure of Land et al (1) was used in the cDNA library constructions because this is the best procedure for obtaining full-length cDNA inserts.

Several approaches have been used to screen the libraries for those clones which possess M-VSG coding sequences. The first approach was to screen the libraries via the Grunstein and Hogness technique (2) with [<sup>32</sup>P]-first strand cDNA from both day 5 population mRNA and from WRTAT 3 mRNA (WRTAT 3 is a *T. rhodesiense* bloodstream clone). This was a "+/- screen" in that clones which hybridized to the day 5 cDNA and not to the WRTAT 3 cDNA were suspected to contain M-VSG coding sequences. A number of clones were identified by this differential hybridization result. Five such clones that have been more fully characterized are called 2L11, 9L9, 6R25, 3L41 and 11R45; the nomenclature refers to the position of the microtitre well in which the bacterial transformant containing the recombinant plasmid is stored.

All, or a substantial portion, of each of these five cDNA sequences has been determined. Four of them (3L41 is the exception) contain sequences which are very similar to the C-terminal homology regions of bloodstream VSGs that have been determined by either our lab or other labs (3, 4). This clearly means that these four are VSG cDNAs but does not prove that they are M-VSG cDNAs; they could be early bloodstream VSG cDNAs. To demonstrate conclusively that they are M-VSGs, another test must be devised.

The obvious proof is to demonstrate either that (i) the cDNA will hybrid-select a mRNA that directs the in vitro translation of a VSG that precipitates with a M-VSG monoclonal (RNA selection) or that (ii) the denatured cDNA will hybridize to a M-VSG mRNA and prevent it from being a template for in vitro translation (in vitro hybrid arrest). Allison Ficht, a post-doc in my lab, has spent the past year almost exclusively on trying to get one or both of these techniques to work. She has been very successful in showing that both techniques work nicely in control experiments using a bloodstream VSG mRNA and polyvalent antisera against it. Unfortunately, and despite a herculean effort, she has not been able to use either technique to show beyond a doubt that one of the 5 cDNAs contains sequences coding for a M-VSG. At the moment we are at a loss to explain why these experiments failed but several feasible technical explanations exist. One is the ever present problem of not enough mRNA to do a large scale experiment. From each day 5 population we only get a few µg of polyA<sup>+</sup> RNA of which only about 5% is M-VSG mRNA. Furthermore if the M-VSG is a minor species it may represent such a small amount of RNA that we won't be able to detect it in either experimental technique. The other main question mark is that some of the monoclonal antibodies may not recognize efficiently an in vitro VSG translation product containing both a signal peptide and a hydrophobic tail that are not present on the mature VSG. So after being confronted with these problems, we turned to two other identification approaches -- RNA dot blots and expression of the cDNA in bacteria.

For the RNA dot blots we utilized mRNA from the day 5 populations shown in Table 1. In each case mRNA was serially diluted and spotted on large square pieces



of nitrocellulose filters for hybridization with the different cDNA probes. Figure 1 shows an example of one such RNA dot blot.

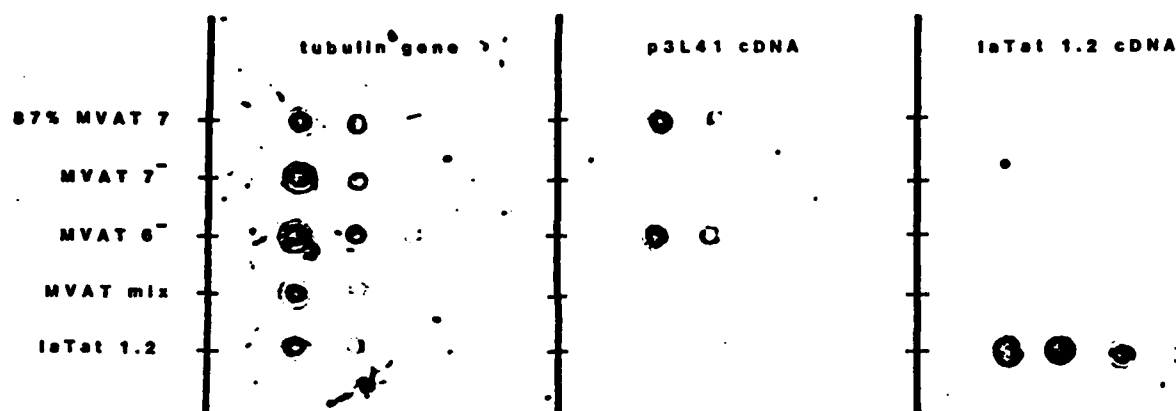


Figure 1 - Autoradiogram of an RNA Dot Blot with three different hybridization probes.

The mRNAs from the four different day 5 populations and a bloodstream *T. brucei* clone, IaTat 1.2, were bound to filters and probed with either a trypanosome tubulin gene, the IaTat 1.2 cDNA or plasmid 3L41. Hybridization to the tubulin gene served as a control to demonstrate that approximately the same amount of RNA from each population had been spotted. Likewise, hybridization of IaTat 1.2 cDNA to only IaTat 1.2 mRNA was a control to show that the hybridization was working properly. Then in the actual experiment (middle panel of Figure 5) p3L41 hybridized strongly to the RNA from the 87% MVAT 7 and MVAT 6 and more weakly to RNA from the MVAT MIX. No hybridization to the RNA in the MVAT 7 and IaTat 1.2 populations was detected. An inspection of Table 1 shows that this result is consistent with p3L41 containing the coding sequence of the MVAT 7 VSG. When p11R45 is used as the hybridization, the result is identical to that obtained with p3L41 (not shown). This suggests that p11R45 also contains the coding sequence of MVAT 7 VSG. The cDNA inserts of 3L41 and 11R45 are each about 700 bp in length. We have determined about 90% of the sequence of each and there is no overlap in the two sequences. However 11R45 contains the 3' polyA<sup>+</sup> tail of the mRNA and a sequence of resembling that of a VSG C-terminal homology region. Therefore, it is clearly a VSG cDNA. The 3L41 cDNA contains one open translation reading frame throughout the entire sequence so must arise from the middle of the coding sequence. Since the VSG mRNA lengths are 1600-1800 nucleotides, it seems likely that 3L41 and 11R45 contain different regions of the same VSG mRNA species as illustrated in Figure 2.

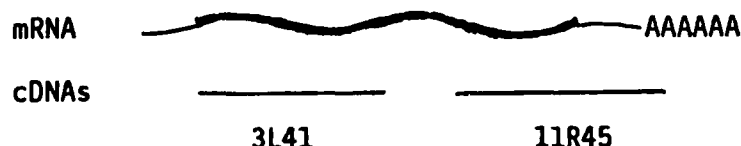


Figure 2. Probable locations of the cDNA sequences relative to the MVAT 7 VSG mRNA

It should be emphasized that the model shown in Figure 2 must still be proven by identifying a third cDNA clone which spans the gap between 3L41 and 11R45.

When RNA dot blots similar to that shown in Figure 1 are performed with the other plasmids identified by the +/- screen and which contain a C-terminal homology sequence, i.e., 2L11, 9L9 and 6R25, a negative result is obtained. They all hybridize weakly to the MVAT MIX RNA but their hybridization to the other day 5 population RNAs is not consistent with their containing the sequence for either MVAT 6 or MVAT 7. They must code for either another of the M-VSGs or for a bloodstream VSG. We are now trying to determine which of these alternatives are correct. For several technical reasons too detailed to go into here, we think 2L11 most likely codes for MVAT 4. To demonstrate this, Klaus Esser is now generating a day 5 population depleted in all M-VSGs except MVAT 4. RNA from this population can then be used to perform a RNA dot blot to confirm that MVAT 4 mRNA corresponds to 2L11. Likewise the generation of other day 5 populations depleted in all but one MVAT can be used to identify the other MVAT cDNAs. We are inclined to think all three specify M-VSGs rather than bloodstream VSGs and anticipate that we'll be able to demonstrate this in the next few months.

Figure 3 shows the DNA sequence of 11R45 in the C-terminal homology region and the 3'-nontranslated region. Although the sequence is not quite complete, an interesting feature has arisen.



Figure 3. Partial sequence at the 3'-end of plasmid 11R45. Arrows show the cysteine residues. The vertical bar is the hydrophobic tail cleavage site. The symbol \* indicates the termination codon.

The C-terminal homology region of 11R45 is somewhat different than that of all bloodstream VSGs that have been sequenced to date. Four cysteine residues are clustered near the C-terminus as in bloodstream VSGs but the amino acids between the cysteines are not nearly as rich in hydrophilic side chains as all sequenced bloodstream VSGs. For example, in bloodstream VSGs the conserved cysteines are almost always followed by lysines. In 11R45, none of the cysteines are followed by lysines. In addition the homology subgroup that 11R45 closely resembles in the hydrophobic tail sequence contains 8 cysteines rather than 4 cysteines as does 11R45. Therefore this M-VSG contains fewer cysteines than expected and fewer charged residues near the C-terminus. In contrast to these differences, the 11R45

sequence does code for a very similar hydrophobic tail cleavage site and a nearly identical hydrophobic tail sequence to that of one of the two homology subgroups. It is not really possible to interpret these findings yet but one interesting possibility is that the M-VSGs go across the trypanosome membrane by the same mechanism as do the bloodstream VSGs but, once across, they either fold up differently or associate with outer membrane surface differently than the bloodstream VSGs. Sequence determinations of other M-VSGs as proposed below will provide more data on this possibility.

Despite the very clear RNA dot blot analysis of 3L41 and 11R45 (Figure 1), there is still a slight possibility that these plasmids do NOT contain the VSG coding sequences recognized by the monoclonals that define the MVAT 7 VSG. To confirm this beyond a doubt, we have turned to expression in bacteria as the identification approach since the RNA selection and hybrid-arrested translation did not work (see above). Even if these techniques had worked we still would need to use bacterial expression to identify the minor M-VSG cDNAs since there is a much lower percentage of these mRNAs in the day 5 population (0.1% or less).

After some false starts, the bacterial expression vector that we are now using is one described by Gray et al (5) in which the foreign DNA fragment to be expressed is inserted into the very last part of the *E. coli*  $\beta$ -galactosidase gene, i.e., 16 codons from the end. This gene is on a plasmid and insertion of the fragment in the correct translation reading frame results a fusion protein of  $\beta$ -galactosidase and the foreign protein. The fusion protein is usually several percent of the total protein of the cell. Furthermore, the  $\beta$ -galactosidase portion of the fusion protein seems to prevent degradation of the foreign portion by bacterial proteases. And finally, there is a nice color selection procedure for identifying colonies possessing the foreign DNA inserted in the right reading frame. The disadvantage of this expression vector is that if the cDNA is full-length, the 5' non-translated region must usually be shaved off for the fusion protein to be synthesized. Despite this modest reservation, this expression system is now working very nicely in our hands.

As of this writing, we have inserted the coding regions of 2L11, 9L9, 6R25 and 3L41 into this vector and demonstrated that fusion proteins are synthesized in each case which can be detected by Commassie blue staining of an SDS-gel of the crude bacterial lysate. We are just beginning this week to perform Western blots on the gels to see if any of the metacyclic monoclonal antibodies can recognize the fusion protein. Furthermore, since the fusion protein possesses most of the  $\beta$ -galactosidase protein, the isolation procedure for  $\beta$ -galactosidase can be used to purify the fusion protein in its native configuration. This protein can then be obtained in sufficient quantities for use in immunological studies on live animals.

We are also now performing genomic DNA Southern experiments using the 5 cDNAs discussed above. We have some autoradiograms that suggest that the M-VSG gene corresponding to 11R45 (MVAT7) is located near a telomere (i.e., the cDNA hybridizes to different DNA fragments in different trypanosome clones) but the results are still tentative. Again, we should have more definitive results in a few weeks.

In summary, progress has not been as rapid during the past year as I had originally expected. Recombinant DNA techniques are now reasonably straightforward for a lab as experienced as ours. Therefore, I anticipated that we would have more results at the end of this first year on this metacyclic project. Indeed, construction of the metacyclic cDNA libraries and identification of potential clones by +/- screening proceeded fairly quickly. But we bogged down considerably in the use of in vitro translations and the monoclonal immunoprecipitations to prove that a given cDNA possessed coding sequences for a specific M-VSG. This

forced us eventually to turn to the RNA dot blots and expression vectors for the identification which are just as reliable but we lost many months in making the transition to these alternative approaches. Nevertheless, we have 5 different cDNAs which code for 4 different VSGs, at least one, and probably all, of which are M-VSGs. Therefore the next year should be quite exciting in terms of analyzing the M-VSG amino acid sequences, their genes, and their mechanism of expression. In addition, we should be able to generate large quantities of the M-VSGs from the expression vectors for study.

#### b. The Bloodstream VSG Gene Project.

To detect features of the M-VSGs and their gene expression that are distinct from the bloodstream VSGs, it is necessary to also characterize the bloodstream VSG genes as well. Therefore, Bill Murphy, David Dorfman and Steve Brentano, graduate students in my lab, have been studying the gene system for IaTat 1.2 VSG. This VSG is expressed in the bloodstream via the ELC mechanism. The trypanosome genome has two very closely related BC genes for this VSG and we have carefully characterized the organization and sequences of the two BC genes and the corresponding ELC. Figure 4 shows a simplified restriction enzyme map of these three regions.

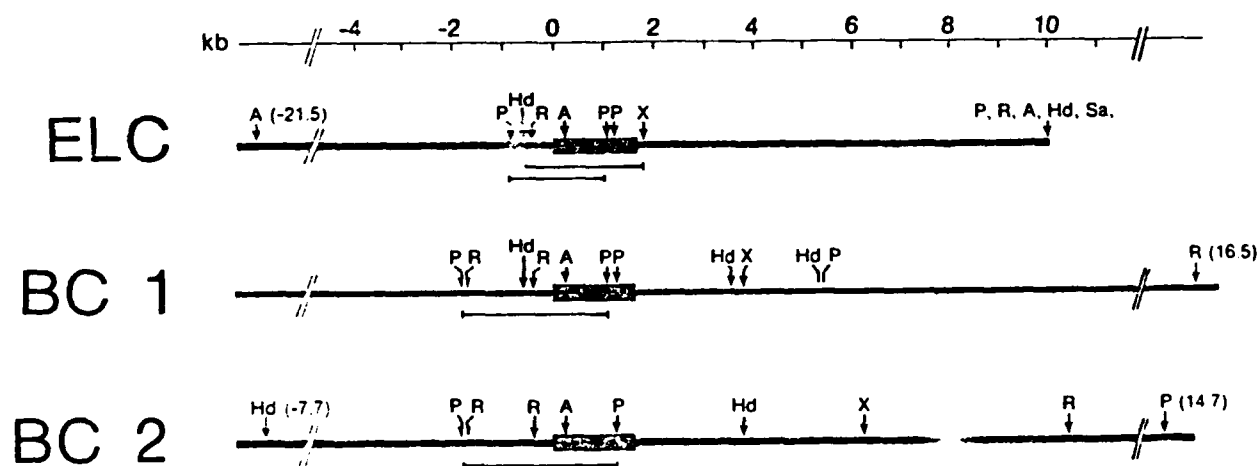


Figure 4 - The three genomic regions to which IaTat 1.2 cDNA hybridizes. Note the telomere downstream from the ELC which mimics cleavage sites for all restriction enzymes. The short horizontal lines indicate regions that were cloned into pBR322.

Sequence determination revealed that the ELC and BC1 coding sequences were identical while BC2 differed in several places (6, 7). This demonstrated that the ELC arose from a duplication of BC1. The regions flanking the ELC and BC1 were determined and from a comparison of these regions, the cross-over points of the duplicated ELC segment were identified. From this information a model was proposed to account for the molecular mechanism involved in the generation of the ELC (8).

It turns out that the early developmental stages of the trypanosome in the midgut of the tsetse fly can be mimicked by establishing bloodstream trypanosomes in a defined culture medium. Under these "procyclic" culture conditions, the bloodstream trypanosomes lose their VSG and their ability to re-infect mammalian hosts within a few days (9, 10). We established IaTat 1.2 trypanosomes in procyclic culture and examined the fate of the ELC gene described above. We expected the ELC gene to be lost concomitantly with the disappearance of VSG mRNA since this would seem the most consistent with the mechanism for turn-off of ELC expression observed in the bloodstream. Surprisingly, this was not the result. The ELC gene remained in the genome of trypanosomes which had been in culture for 40 days even though the VSG mRNA disappeared within the first day or two (7, 8). Thus the ELC is still present in procyclic organisms but its transcription product is not. This suggests that there must be additional mechanisms that control the expression of the ELC gene. At this stage the details of these additional mechanisms are unclear. However trypanosomes undergo many morphological and metabolic changes in the transition from bloodstream form to procyclic forms, any one of which might be involved in the regulation of VSG gene transcription. Two of many possibilities are metabolite repression or temperature inactivation of a transcription factor. Clearly, much more work needs to be done to distinguish between these and other possibilities.

This retention of the ELC and loss of VSG mRNA in procyclic culture have some interesting implications for the above metacyclic VSG gene project. Hadjuk and Vickerman (11) have reported that when bloodstream trypanosomes expressing a given VSG are passaged through tsetse flies which are then allowed to bite an uninfected animal, the first new wave of bloodstream trypanosomes usually expresses the same VSG as that ingested by the tsetse fly. This suggests that an ELC gene being expressed at the time of the initial tsetse fly bite remains in its expression site, but turned off, during passage through the fly and is available for continued expression upon re-introduction of the parasite into the bloodstream. It would also seem to imply that expression of metacyclic VSG genes occurs either from a different site or in such a way that it does not disturb the ELC already in place. We should soon be in a position to perform experiments designed to test these possibilities.

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